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**Glucosinolates, myrosinase hydrolysis products and flavonols found in rocket (*Eruca sativa* and *Diplotaxis tenuifolia*)**

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## Abstract

Rocket species have been shown to have very high concentrations of glucosinolates and flavonols, which have numerous positive health benefits with regular consumption. In this review we highlight how breeders and processors of rocket species can utilize genomic and phytochemical research to improve varieties and enhance the nutritive benefits to consumers. Plant breeders are increasingly looking to new technologies such as HPLC, UPLC, LC-MS and GC-MS to screen populations for their phytochemical content to inform plant selections. Here we collate the research that has been conducted to-date in rocket, and summarise all glucosinolate and flavonol compounds identified in the species. We emphasize the importance of the broad screening of populations for phytochemicals and myrosinase degradation products, as well as unique traits that may be found in underutilized gene bank resources. We also stress that collaboration with industrial partners is becoming essential for long-term plant breeding goals through research.

**Key words:** *Brassicaceae*, Isothiocyanates, Plant breeding, Indoles, Nitriles

## 26 **Introduction**

27 In recent years, several species of minor leafy-crops have risen to prominence as potentially  
28 important commercial and edible species. One example is rocket, which has quickly gained  
29 popularity in the Western diet. Originally found as an obscure crop in Mediterranean and  
30 Middle-Eastern countries, rocket has become popular largely due to the pungent aromas and  
31 tastes associated with it. Glucosinolates (GSLs)/isothiocyanates (ITCs) and flavonols derived  
32 from many species <sup>1-4</sup> have been shown to confer significant protection against cancer and  
33 heart disease <sup>4-11</sup>. In Western countries, diets are generally lacking in fruits and vegetables.  
34 Despite government initiatives (such as the “5-a-day” campaign in the UK and USA), these  
35 diseases are increasingly leading to premature deaths <sup>12</sup>. Plant breeders aim to maximize  
36 levels of such beneficial compounds, but with little genomic information about rocket species  
37 presently available, this is a formidable task. This review will give an overview of research in  
38 rocket, an outbreeding crop, and how breeders and processors can utilize it to enhance  
39 beneficial compounds.

## 40 **Rocket species**

41 Rocket (also known as arugula, rucola and roquette) is a leafy vegetable crop that has gained  
42 substantial popularity across the world, particularly over the last fifteen years <sup>13-16</sup>. Two main  
43 species are predominantly farmed as salad crops; these are *Eruca sativa* (‘salad’ or ‘cultivated’  
44 rocket; sometimes referred to as *Eruca vesicaria* subsp. *sativa*) and *Diplotaxis tenuifolia* (‘wild’  
45 rocket). Both species share a peppery taste and aroma that is very distinctive <sup>17</sup>. They have  
46 been reported to contain high levels of vitamin C, GSLs, flavonols and phenolics <sup>18-25</sup>. These  
47 are all known to have both anti-oxidant and anti-cancer properties, and are also implicated in  
48 lowering the risk of cardiovascular and cognitive disease. For excellent information on these  
49 beneficial effects and their underlying causes, see Drewnowski & Gomez-Carneros <sup>26</sup>, Keum

et.al <sup>27</sup>, D'Antuono et.al <sup>28</sup>, Egea-Gilbert et.al <sup>29</sup>, Degl'Innoocenti et.al <sup>30</sup>, Bjorkman et.al <sup>31</sup> and Jeffery et.al <sup>32</sup>.

## **Taxonomy and domestication**

A distinction should be made that both *Eruca* and *Diplotaxis* species have overlapping characteristics, and that one can be easily mistaken for the other by the untrained eye, and/or before a certain level of maturity has been reached <sup>28</sup>. It is also arguable that *D. tenuifolia* is the least 'wild' of the two species even though the common name is 'wild rocket'. It is featured and favored in commercial products and breeding programs, and is likely to be more domesticated than *Eruca* species as a result. *Diplotaxis* varieties are generally uniform phenotypically, with *Eruca* varieties being more diverse in this respect <sup>23</sup>. No direct genomic evidence has been presented in the literature to suggest one species is any more or less genetically variable than the other. Variability in GSL data seems to support the hypothesis that *Diplotaxis* species are more 'wild' <sup>33</sup>, though it is not conclusive, as only a relatively small number of cultivars have been tested. This is a point that needs clarification through research and extensive breeding, as neither species can be considered fully domesticated <sup>29</sup>. For example, germination rates are variable, reproductive organs are typically small, seedpods shatter and disperse freely (rather than staying on the plant), and physical defenses such as leaf hairs are still present in many commercial varieties <sup>34</sup>.

## **Phytochemicals in *Eruca sativa* and *Diplotaxis tenuifolia*: types and structures**

### **Glucosinolates**

GSLs are  $\beta$ -thioglucoside *N*-hydrosulphates that are responsible for the sharp and bitter-tasting flavors found in cruciferous vegetables <sup>35,36</sup>. In combination with the enzyme myrosinase (thioglucoside glucohydrolase, EC 3.2.1.147), GSLs are hydrolyzed to create isothiocyanates, nitriles, thiocyanates, epithionitriles, indoles, oxazolidine-2-thiones,

75 cyanopithioalkanes, ascorbigens, goitrogens and epithioalkanes <sup>37-49</sup>; see Figure 1. Many of  
76 these hydrolysis products have antibacterial, antifungal and insect repellent effects <sup>50-55</sup>. GSLs  
77 and ITCs are being increasingly used as 'biofumigants' to suppress soil borne pathogens,  
78 nematodes and weeds. Some of the volatile products have the opposite effect of attracting  
79 species that can tolerate high GSL concentrations, such as types of ovipositing insect <sup>56,57</sup>.

80 The conditions under which hydrolysis of GSLs occurs will affect the respective  
81 proportions of the chemicals produced; pH, iron ions, thiol ions, temperature and hydration  
82 play a particularly prominent role in this process *in vivo* <sup>58</sup>. The separation of GSLs in  
83 specialist 'S-cells' from myrosinase in myrosin cells means that the two components only  
84 come into contact upon tissue disruption; for example when damaged via chewing or  
85 digestion <sup>59-69</sup>. It is the biological activity of the ITC hydrolysis products in humans that are of  
86 most interest in rocket <sup>50</sup>. GSLs can be hydrolyzed within the intestinal tract by gut microflora  
87 that are known to have specific myrosinase activity <sup>70-73</sup>, but the efficacy of their action is not  
88 yet well determined.

89 GSL concentrations can vary and change over time depending on environmental  
90 conditions and stress <sup>7</sup>. Other factors affecting GSL profiles include the plant age, organ type,  
91 developmental stage, ambient air temperature, level of water stress, photoperiod, agronomic  
92 practice, degree of wounding, and geographical origin of the variety/species <sup>74-81</sup>. These can  
93 often affect the profiles of *all* phytonutrients contained within tissue, not just GSLs <sup>82</sup>, and they  
94 are all factors that plant breeders aim to mitigate through development of genetically  
95 advanced and uniform breeding lines.

96 GSLs and the ITC derivatives have been an integral part of the human diet for millennia  
97 because of the presence of them in the family *Brassicaceae* <sup>64-66,83-89</sup>. GSLs are evolutionarily  
98 recent secondary metabolic products having arisen 10-15 million years ago <sup>90,91</sup>, acting to  
99 prevent pathogen attack and dissuade herbivory. They are known in only a few angiosperm

100 families of the order *Brassicales*, which includes the *Brassicaceae* <sup>92-100</sup>, and of which *Eruca*  
101 and *Diplotaxis* are members.

102 A study by Pasini et al. <sup>33</sup> of 37 rocket accessions (*Diplotaxis* and *Eruca*) showed that  
103 GSL profiles were all very similar, regardless of the species. In total, twelve GSL compounds  
104 were found across all accessions; Table 1 illustrates all known GSL compounds identified to-  
105 date in rocket. These include 4-mercaptobutyl GSL (glucosativin) <sup>21</sup>, 4-methylthiobutyl GSL  
106 (glucoerucin) <sup>101</sup>, and 4-methylsulfinylbutyl GSL (glucoraphanin) <sup>28</sup>, which constitute the  
107 three most abundant GSLs in rocket.

## 108 **Flavonols**

109 Flavonols are diphenylpropanes (C<sub>6</sub>-C<sub>3</sub>-C<sub>6</sub>) <sup>102</sup> and are another important group of chemicals  
110 found within rocket species. Flavonols in rocket are found with sugar conjugates, and  
111 typically occur in relatively large quantities <sup>103</sup>. The aglycones found (such as quercetin and  
112 kaempferol) are glycosylated and acylated, which in turn affects their biological properties <sup>18</sup>.  
113 A study by Martínez-Sánchez et al. <sup>18</sup> identified over 50 different flavonol compounds across  
114 four different species. Watercress, mizuna and two species of rocket were all found to  
115 accumulate very different compounds within their leaves, and in varying quantities. Wild  
116 rocket showed high levels of quercetin-3,3',4-triglucosyl (43.5 mg per 100g<sup>-1</sup> fw) and salad  
117 rocket had mostly kaempferol-3,4'-diglucosyl (97.8mg per 100g<sup>-1</sup> fw). The group also showed  
118 a correlation between quercetin derivatives and high antioxidant activity, despite the  
119 significant variations seen between species.

120 Studies conducted on rocket tissues have identified significant concentrations of  
121 polyglycosylated flavonols. The core aglycones of these are kaempferol, quercetin and  
122 isorhamnetin <sup>102</sup>; Table 2 provides an up-to-date list of all flavonol compounds identified in  
123 rocket to-date. Martinez-Sanchez et al. <sup>18</sup> showed that *Eruca* species accumulate kaempferol  
124 derivatives, whereas *D. tenuifolia* accumulates predominantly quercetin instead, meaning that



125 the two chemicals could be used as an identification marker between the two species <sup>104</sup>.  
126 Isorhamnetin aglycones are common to both species but typically in much lower  
127 concentrations <sup>33</sup>. The specific aglycones also infer varying degrees of anti-oxidant activity.  
128 For example, quercetin derivatives have a higher activity than kaempferol and isorhamnetin.  
129 The differences in structure (the arrangement of hydroxyl groups and glycosylation) affect  
130 anti-oxidant activity by allowing the molecules to act as hydrogen/electron donors, single  
131 oxygen scavengers, or as reducing agents <sup>105</sup>.

### 132 133 **Phytochemicals and the relation with quality: taste and aroma**

134 It is thought that the presence of glucosativin, glucoerucin and their hydrolysis products  
135 within rocket leaves is what gives them a characteristic flavor <sup>44</sup>. Many of the health beneficial  
136 GSLs and ITCs are thought to be responsible for strong tastes that *some* consumers find  
137 repellant <sup>106</sup>. It seems that to many people, these compounds contribute very little to a  
138 pleasurable eating experience and are actively avoided <sup>83</sup>. Conversely however, some people  
139 *do* prefer these strong tastes and aromas, and will actively seek to consume rocket when it is  
140 available. Growers in Italy often prefer the subsequent cuts because of the more intense tastes  
141 and aromas that are produced <sup>107</sup> and some will even ‘sacrifice’ the first cut in favour of the  
142 subsequent leaf growth. This highlights a divide between consumers that may be indicative of  
143 underlying genotype(s) for taste perception and preference.

144 The breeding process in rocket varieties to-date has effectively made the species  
145 ‘milder’ in taste when compared to plants that grow naturally in the wild. Whether this has  
146 been intentional or as a result of selecting for other unrelated traits (such as leaf morphology)  
147 is debatable. Some recent commercial varieties have been bred for a ‘hotter’ taste, such as  
148 ‘*Wildfire*’, by Tozer Seeds (Surrey, UK).

149 A study by Pasini et al. <sup>17</sup> demonstrated how breeding for sensory traits could be  
150 achieved, by highlighting which glucosinolates contributed to specific taste and aroma  
151 elements in rocket. It was found that progoitrin/epiprogoitrin is responsible for bitter taste  
152 attributes, despite being only a minor component of the overall GSL profile of rocket (4.3-  
153 11.4% of total GSL concentration). The perceived pungency of leaves was positively related to  
154 the overall GSL content of accessions, and the levels of glucoraphanin negatively contributed  
155 to the typical 'rocket' flavour. The study also highlighted an important difference between  
156 rocket and other *Brassica* sensory studies <sup>108</sup>, in that bitterness was perceived as a favorable  
157 characteristic according to panelists. The flavonol compound kaempferol-3-(2-sinapoyl-  
158 glucoside)-4'-glucoside also significantly and positively contributed to flavor attributes in  
159 *Eruca* accessions. This would indicate that GSL compounds are not totally responsible for  
160 flavor in rocket. The study itself stopped short of saying how or if the information obtained  
161 would be used in breeding programs, but with study into rocket flavor components, milder  
162 (and/or stronger) varieties could be bred more efficiently once the responsible compounds  
163 are properly identified <sup>26</sup>.

164

## 165 **Health promoting properties of glucosinolate-myrosinase products and flavonols of** 166 **rocket**

### 167 **Isothiocyanates**

168 ITC hydrolysis products have been identified in rocket <sup>45</sup>, such as 4-(methylthio)butyl ITC  
169 (erucin) <sup>109,110</sup> which is known to show anti-proliferative activity in human lung carcinoma  
170 A549 cells, hepatoma (HepG2) cells, colon cancer cells, prostate cancer cell lines (PC3, BPH-1  
171 and LnCap) and leukemia cells <sup>111</sup>. Erucin is a structurally reduced analog of sulforaphane,  
172 (which is predominantly found in broccoli) and has shown promising anti-cancer properties  
173 *in vitro* (e.g. anti-proliferation of human erytroleukemic K562 cells) <sup>112</sup>. Research into the

174 chemopreventative and anti-genotoxic nature of ITCs has shown promising results <sup>113</sup> (see  
175 Figure 2). Other studies involving chemically induced genotoxicity have shown very strong  
176 anti-genotoxic effects of *E. sativa* extracts <sup>13</sup> which is in agreement with other *Brassicaceae*  
177 studies <sup>114,115</sup>. Identifying specific cultivars of rocket with elevated levels of erucin and  
178 glucoraphanin would be an important first-step in developing superior varieties from a  
179 human nutrition standpoint.

180         The results of GSL/ITC research prompted an investment in broccoli breeding in the  
181 last decade. A similar concerted effort could be made for rocket which contains similar  
182 compounds, and which are potentially just as efficacious in humans <sup>116</sup>. Erucin for example,  
183 has been shown to have very similar, and even superior, biological activity to sulforaphane <sup>117</sup>.  
184 One paper has specifically demonstrated that the concentrations of rocket ingested in an  
185 average daily diet is significant enough to infer a cancer preventative effect <sup>13</sup>. The  
186 metabolism of ITCs in humans via the mercapturic acid pathway has been investigated. ITCs  
187 are conjugated with glutathione and degraded by N-acetylation, initiating an increase of phase  
188 II detoxification enzymes; see Figure 3 for detailed pathway breakdown of erucin <sup>113</sup>.

## 189 **Nitriles**

190 Along with ITCs, nitriles are the most abundant bioactive compounds produced by GSL  
191 hydrolysis <sup>116</sup>. The hydrolysis of glucoraphanin for example, yields predominantly  
192 sulforaphane and sulforaphane nitrile. The ratio in which the two are formed depends greatly  
193 upon the environmental conditions and the plant cultivar that is used <sup>117</sup>. A low pH medium  
194 tends towards the formation of nitriles, whereas high pH forms ITCs <sup>118,119</sup>. The presence of  
195 thiol and iron ions favors nitriles, and high temperature and hydration produce more ITCs  
196 <sup>120,121</sup>. This can have substantial consequences for any potential health benefits that might be  
197 inferred from eating rocket <sup>119</sup>. The nitrile form is approximately three orders of magnitude  
198 less efficacious than the ITC in inducing quinone reductase (phase II enzyme), and thus infers

199 a reduced enzymatic and anticarcinogenic response. Nitriles also compete with ITCs in this  
200 induction, and reduce potential positive effects further <sup>38</sup>. As the ratio of these compounds  
201 may depend on plant variety, care must be taken in rocket breeding when selecting plants for  
202 GSL content, as this may not be reflective of the bioactives produced in subsequent hydrolysis  
203 reactions <sup>122</sup>. Other underlying genetic factors may influence which degradation pathway is  
204 taken.

## 205 **Indoles**

206 Indoles are the predominant autolysis product of indole glucosinolates such as glucobrassicin,  
207 as their ITC counterparts are unstable <sup>85</sup>. Glucobrassicin has been detected as a minor GSL in  
208 rocket species <sup>33</sup>, and the predominant indole species produced is indole-3-carbinol. This  
209 compound is known to be cancer-preventative <sup>125,126</sup>, particularly in reproductive organs *in*  
210 *vitro* and *in vivo*. A condensation product of indole-3-carbinol, 3,3'-diindolymethane, is also  
211 responsible for beneficial physiological effects. Both compounds have been shown to reduce  
212 cell proliferation in breast, prostate, cervical and colon cancer cell lines. They also show  
213 distinct differences from ITCs such as sulforaphane <sup>127</sup>, and inhibition of tumor development  
214 in the stomach, breast, uterus, tongue and liver of rodents <sup>128-135</sup>. Experiments in rodents have  
215 shown an increase in drug-metabolizing enzymes in the stomach, liver and small intestines of  
216 individuals consuming both ITCs and indoles. This is suggestive of enhanced detoxification  
217 phase II enzymes (such as quinone reductase, glutathione reductase and glutathione  
218 transferase) <sup>134</sup>, and a mechanism by which these phytochemicals infer chemopreventative  
219 effects <sup>135,136</sup>.

220 Typically indoles inhibit cell proliferation through cytostatic mechanisms, whereas  
221 ITCs induce cytotoxicity within cell lines (at above 12.5 $\mu$ M concentrations), which ultimately  
222 leads to increased apoptosis <sup>137,138</sup>. This indicates that both types of compound could act and  
223 be effective at different stages of cancer development <sup>11</sup>. Indoles have been shown to induce

224 programmed cell death in prostate, breast and osteocarcinoma cell lines <sup>139</sup> and G<sub>1</sub> cell cycle  
225 arrest in breast and prostate cancer cell lines <sup>142,143</sup>. It is these cytostatic effects on cell  
226 proliferation that has been suggested as the mechanism responsible for the lack of apoptosis  
227 effects in indoles <sup>141</sup>.

228 Using information on GSL content in rocket, the ITC and indole effects can be  
229 potentially maximized in new varieties, and be of a greater benefit to human health when  
230 considered in tandem, rather than separately <sup>127</sup>.

### 231 **Oxazolidine-2-thiones & goitrogens**

232 The hydrolysis of  $\beta$ -hydroxy-alkyl GSL compounds (e.g. progoitrin; a minor GSL in rocket) can  
233 produce oxazolidine-2-thiones such as goitrin (5-vinyloxazolidine-2-thione) <sup>142-148</sup>. It is these  
234 compounds that are largely attributed to the thyroid condition of goiter in mammals <sup>149</sup>, but  
235 the action of microflora in the gut is thought to mediate the problems associated with high  
236 oxazolidine-2-thione intake <sup>150,151</sup>. That being said, oxazolidine-2-thiones interfere with  
237 thyroxine synthesis <sup>154</sup> and are therefore likely to have an adverse biological effect regardless  
238 of gut microflora action or bodily iodine status <sup>3</sup>. A study by Nishie and Daxenbilcher <sup>155</sup>  
239 showed that these compounds are not teratogenic or embryotoxic however.

240 These molecules contribute significantly to the bitter taste of rocket that some people  
241 perceive quite strongly <sup>154</sup>. The detection of these compounds may be mediated in a similar  
242 genetic fashion as PROP (propylthiouracil), for example <sup>155,156</sup>. By using phytochemical data in  
243 rocket breeding programs these oxazolidine-2-thione components could be reduced,  
244 potentially improving consumer acceptance (depending on the target consumer) and avoiding  
245 any possible adverse health effects associated with over-consumption.

### 246 **Ascorbigens**

247 Ascorbigens are formed via the reaction of indole-3-carbinol and 3,3'-diindolymethane with  
248 ascorbic acid in the stomach during myrosinase-catalyzed degradation of indoly-3methyl

glucosinolates <sup>157,158</sup>. In this manner it is thought that ascorbigens have a role in cancer-modulation <sup>159</sup> via quinone reductase induction <sup>114</sup>. As has been highlighted previously, this has important implications for breeding for plant varieties with enhanced chemopreventative effects.

### **Epithioalkanes**

Epithioalkanes are formed as part of the myrosinase reaction with GSLs at low pH with epithiospecifier protein and ferrous ions. These GSLs typically have a side-chain with a double bond, such as sinigrin <sup>160,161</sup>. It is uncertain whether these compounds produce any significant bioactive effect in humans, but the ratio in which they are produced alongside ITCs, nitriles and indoles may impact on these compounds' efficacy as anti-carcinogens.

### **Flavonols**

The antioxidant and anti-inflammatory function of flavonols in the human diet are well known and include protecting the colonic epithelium from free radical damage <sup>164-167</sup>. They can induce the up-regulation of enzymes (such as cytochrome P450), that may lead to a decreased risk of cancer, cardiovascular disease, immune dysfunction, atherosclerosis and chronic inflammation <sup>168,169</sup>.

## **Factors affecting phytochemical content**

### **Breeding and cultivation**

Rocket has been consistently shown to be a good dietary source for flavonols, GSLs and anti-oxidants. However, there can be large differences between plants of the same germplasm accession due to a combination of genetic and environmental variability. This is probably due to the outbreeding nature of the species <sup>104</sup> and a lack of overall uniformity in varieties. Commercial varieties cannot be considered truly domesticated because of this tendency for outcrossing, and the susceptibility of plants to inbreeding depression (a loss of genetic

274 variability due to repeated self-pollination or crossing with a closely related individual).  
275 Development of advanced open-pollinating breeding lines (lines that are allowed to cross-  
276 pollinate freely in a population of selected individuals), or even F<sub>1</sub> hybrids (superior varieties  
277 produced by crossing distinctly different, elite inbred lines), could potentially minimize such  
278 variation.

279       Throughout the food chain there are many aspects that can have an adverse effect on  
280 GSL levels within leaves (Figure 4). These include the cultivar choice, cultivation practice,  
281 climatic conditions, photoperiod, sulphur and nitrogen availability, harvest date, time spent in  
282 storage, the temperature of wash water, levels of physical damage to leaves, packaging  
283 atmosphere and food preparation methods <sup>30-32,170-173</sup>.

#### 284 **Harvesting**

285 Rocket species have the ability to re-grow their leaves repeatedly after cutting, which allows  
286 for several harvests to take place under optimal conditions <sup>107</sup>. In parts of southern Italy, it is  
287 not unheard of for up to seven harvests to occur from a single planting. This has obvious cost-  
288 saving benefits for growers, but multiple harvests also induce stress responses in rocket that  
289 may be detrimental to the flavor and aesthetics of the crop. Stress drives up the production of  
290 secondary metabolites such as GSLs and anthocyanins, which will produce very strong, bitter  
291 tastes. There are other detrimental effects of multiple harvests; leaves become progressively  
292 smaller and more 'skeletal' in appearance with each cutting, for example. High anthocyanin  
293 levels also affect the color of leaves, turning them an undesirable pink, purple or red. Color  
294 has been found to be one of the most important characteristics consumers look for in rocket  
295 <sup>174</sup>, and so the loss of fresh appearance can ultimately lead to rejection of crops by  
296 supermarkets and processors.

#### 297 **Industrial and culinary processing**

298 There are five main influences that have been identified in affecting GSL levels during  
299 processing <sup>94</sup>. These are the action of myrosinase hydrolysis, myrosinase inactivation, the  
300 lysis and leaching of GSLs into wash-water, thermal degradation of GSLs, and the loss of  
301 ascorbic acid, iron and other enzyme co-factors. Myrosinase inactivation and thermal  
302 degradation of GSLs is probably less of an issue in rocket species, as the leaves are not  
303 typically cooked. The leaves are not ordinarily frozen, and so freeze-thaw hydrolysis is not  
304 likely to be a major factor either. Other factors almost certainly play a significant role in GSL  
305 and phytochemical loss in rocket. Verkerk et al. <sup>94</sup> highlighted four key areas that affect GSL  
306 levels before reaching the end consumer. These are:

- 307 1. The variety / cultivar used
- 308 2. Storage and packaging (post-harvest, post-processing & in shops/supermarkets)
- 309 3. Industrial processing
- 310 4. Consumer preparation methods

311 If each of these areas can be mitigated through breeding superior varieties, consumers  
312 will receive an end product that is of higher nutritive quality and thus provide increased  
313 health benefits.

#### 314 **Post harvest storage**

315 Studies on both *Diplotaxis* and *Eruca* species have been conducted to determine the effects of  
316 post harvest storage conditions on chlorophyll content and respiration rates <sup>15</sup>. Both species  
317 of rocket have been found to have high respiration rates <sup>107</sup> leading to rapidly impaired visual  
318 quality, such as stem browning, tissue yellowing and general decay <sup>175</sup>. Provided initial GSL  
319 loss can be mitigated through breeding, ITC formation has been shown to increase over nitrile  
320 formation during the storage period <sup>176</sup>.

321 Time, temperature, humidity and atmospheric conditions are all optimized for specific  
322 crops within the logistics chain, but these factors are often only designed to prevent visual



323 degradation and not phytochemical breakdown <sup>100</sup>. Getting producers, packagers and  
324 transporters to change their current practices in order to better preserve the health-  
325 promoting compounds in rocket would be a difficult task. Treatments and storage conditions  
326 are often integrated parts of protocols and procedures, and changing these would require  
327 significant testing on a commercial scale.

328

## 329 **New selection tools for breeders**

### 330 **Phytochemical selection**

331 It should not be forgotten that some GSLs and their breakdown products are thought to be  
332 toxic, and even carcinogenic, at high concentrations <sup>128</sup>. Breeders and researchers should be  
333 mindful that more of a certain compound does not necessarily mean 'better' <sup>177</sup>. Humans seem  
334 to be able to tolerate GSLs much better than pigs, rats and rabbits for example; but  
335 overconsumption of these compounds may have serious health consequences <sup>64</sup> as high dose-  
336 effect relationships are as yet unknown in humans <sup>94</sup>. Few papers in GSL research (regardless  
337 of species) have acknowledged the potential for plant breeders to utilize HPLC/UPLC/LC-  
338 MS/GC-MS methods within breeding programs to 'monitor' and select plants for their  
339 phytochemical content in this manner. These techniques would provide valuable information  
340 on breeding lines relatively rapidly, especially for GSL and flavonol breeding <sup>178</sup>. It is not  
341 common practice to select rocket plants based on their phytochemical profile at present, but  
342 as interest in these compounds increases it will be necessary for breeders to modify their  
343 selection criteria and information sources in order to remain competitive in the salad  
344 vegetable market <sup>94</sup>. This has been achieved with '*Beneforte*' broccoli (Seminis Vegetable  
345 Seeds; subsidiary of Monsanto Company, St. Louis, Missouri, USA; [www.beneforte.com](http://www.beneforte.com)) for  
346 example. It has also been indicated in hybrid varieties of *Brassica* that ITC/nitrile ratios can be  
347 selected for <sup>179</sup>.

## 348    **Genetic resources and Marker Assisted Breeding**

349    European initiatives (such as the EU GENRES project ‘Leafy vegetables germplasm,  
350    stimulating use’; <http://documents.plant.wur.nl/cgn/pgr/leafyveg/>) have included rocket  
351    species within their remit, indicating the rising prominence of the species, and the desire for  
352    more work to be conducted on them. The germplasm accessions stored in gene banks are a  
353    valuable genetic resource for breeders to take advantage of <sup>180</sup>. The accessions contained  
354    within these collections are highly variable and have unique visual and sensory  
355    characteristics that could be introgressed into breeding lines relatively easily <sup>181</sup>.

356            Genetic information about rocket within the published literature is very scarce. Some  
357    molecular marker techniques such as Random Amplification of Polymorphic DNA (RAPD),  
358    Inter-Simple Sequence Repeats (ISSR) and Amplified Fragment Length Polymorphisms  
359    (AFLP) have been used to analyze morphological characteristics of *Eruca vesicaria* <sup>29</sup>. ISSR and  
360    AFLP are relatively robust for screening variable populations and discriminating between  
361    cultivars <sup>180</sup> but RAPDs are notoriously unreliable and suffer from a lack of reproducibility  
362    and resolution. Perhaps one of the most underutilized marker types is SRAP (Sequence  
363    Related Amplified Polymorphism). The forward and reverse primers are designed to target  
364    arbitrary GC and AT rich sequences of the genome respectively, and are therefore more likely  
365    to anneal to active genomic regions <sup>182</sup>. This could be of use in understudied crops such as  
366    rocket, as it provides a simple, repeatable and reliable way of screening large populations.

367            These techniques are now for the most part however, obsolete in advanced molecular  
368    plant breeding, as NGS (Next Generation Sequencing) and SNP (Single Nucleotide  
369    Polymorphism)/QTL (Quantitative Trait Loci) analyses are far more specific, reliable and  
370    cost-effective. SNPs are the most abundant marker type within genomes, and their high  
371    density is ideal for studying specific regions in detail <sup>183</sup>. NGS techniques are now relatively  
372    affordable, even for relatively small companies. They are widely available in academic

373 institutions, but many companies are bypassing these in favor of dedicated private  
374 commercial services <sup>184</sup> or are developing their own in-house facilities. The inability of some  
375 research institutions to provide adequate customer service, cost-effectiveness, data storage,  
376 and results on time is jeopardizing how much knowledge is in the public domain. Increasingly,  
377 both large and small breeding companies are collaborating privately and advancing  
378 techniques far beyond those found in academic institutions. Future work by institutes in  
379 advanced genomics, sequencing and genotyping is likely to be obsolete in some cases because  
380 private research is already finding new innovations, e.g. for data storage and bioinformatics.  
381 Because private companies have no obligation to share their knowledge, many of these  
382 advances may be unobserved by the mainstream scientific community. Institutes and  
383 Universities need to do more to attract business from industry in order to keep up with the  
384 pace of private advances in this area.

385         Transcriptome sequences are now (generally) adequate for breeders to use and make  
386 huge advances in only a few years. Linkage mapping and QTL analyses can be conducted on  
387 desktop computers, making integration into breeding companies relatively straightforward  
388 from an IT point of view, even if the actual sequencing and genotyping are outsourced. Again,  
389 this may typically be to private companies providing a dedicated service. The availability of  
390 software licenses and advanced training courses from private companies also means plant  
391 breeders do not necessarily need the expertise found in Universities and research institutes in  
392 order to attain their goals.

393

## 394 **Summary**

395 Of all the research papers concerning rocket species and their phytochemistry, none have  
396 directly addressed how information could be used within a working breeding population.  
397 Often it is explained or postulated purely as theory rather than actual practice, or only given a

398 cursory mention. Only very rarely is a plant breeding program reflective of theory, due to the  
399 large number of environmental factors affecting plant growth, development and reproduction.  
400 The progressive selection of rocket plants through conventional/molecular breeding would  
401 be a valuable tool for the research community as well as providing an excellent incentive for  
402 breeding companies to fund research. The actual monitoring and quantification of  
403 GSL/flavonol levels through successive generations (i.e. not just one as has been the case with  
404 most studies) would not only validate the heritability of such traits in rocket, but would also  
405 provide a 'roadmap' for how other minor crops might be developed for commercial use.

406 Attention must be paid to the phytochemical content of varieties within breeding  
407 populations of rocket. By focusing solely on morphological traits, important phytochemical  
408 genotypes may be inadvertently lost from populations; this could be said of all *Brassicaceae*  
409 species, not just rocket. The balance of glucosinolate-myrosinase degradation products does  
410 seem to have a genetic component to it and so could be selected for also. Utilising genetic  
411 resources, the falling costs of sequencing and bioinformatics can produce nutritively superior  
412 varieties of rocket in the near future. Plant breeding typically takes longer than the average  
413 research project allows for, even with the use of advanced genomic selection methods. This is  
414 a situation that could be remedied by long-term industrial collaboration and sponsorship by  
415 plant breeding firms.

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694

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## Figure captions

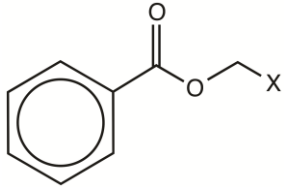
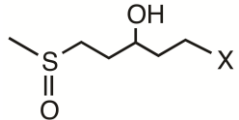
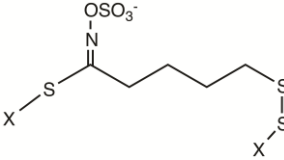
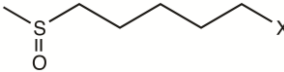
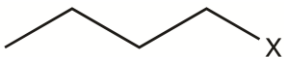
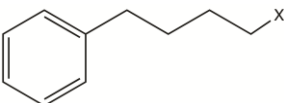
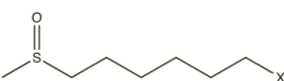
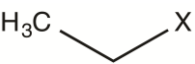
**Figure 1:** – The glucosinolate-myrosinase reaction and some of the subsequent compounds produced under different conditions, such as pH and the influence of epithiospecifier proteins (ESP) (Adapted from Zhang <sup>9</sup> and Hall et al. <sup>185</sup>).

**Figure 2:** – Pathways of documented ITC action in tumorigenic cells. See Wu et al. <sup>113</sup> for a detailed review of the roles ITCs play in cancer prevention.

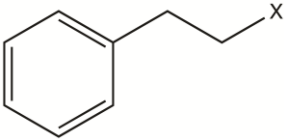
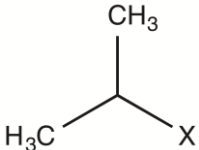
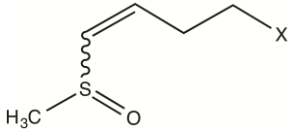
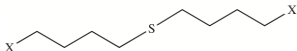
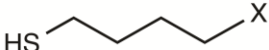
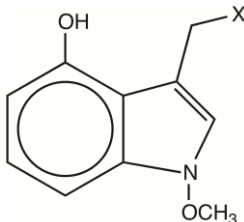
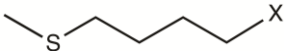
**Figure 3:** – The mercapturic acid pathway of ITC metabolism in the human body. After ingestion of rocket leaves glucoerucin is hydrolyzed by myrosinase to form erucin. This is released and absorbed in the ileum, where it is transported in the blood to cells around the body. ITCs initiate Phase II detoxification enzymes in this pathway, and are known to aid in cancer prevention. (Adapted from Wu et al. <sup>113</sup>).

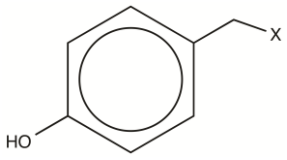
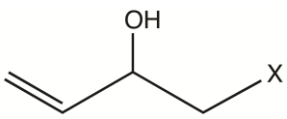
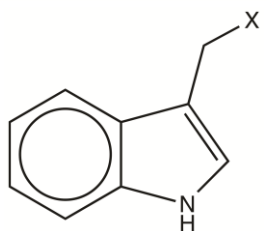
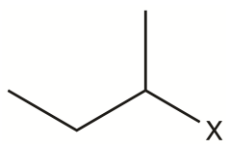
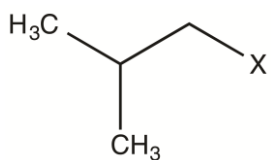
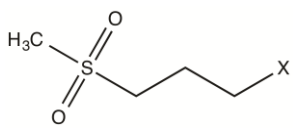
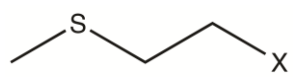
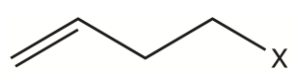
**Figure 4:** – Factors and conditions within the commercial supply chain that affect GSL and flavonol levels within rocket leaves.

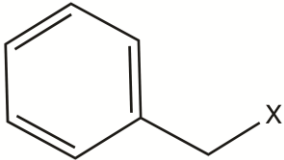
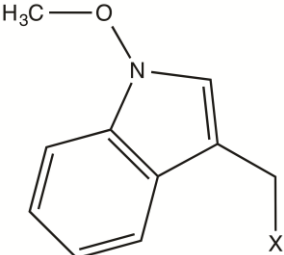
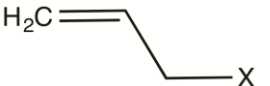
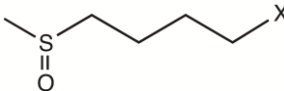
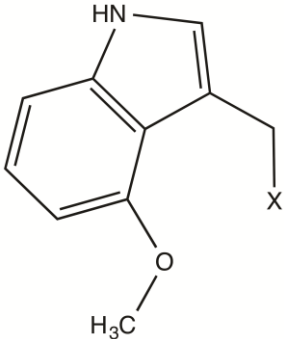
**Table 1:** – Intact Glucosinolates Identified Within Leaves Of Rocket, *Eruca* and *Diplotaxis* Species, By LC-MS (Negative Ion Mode)

| R-group                                | Common name       | R-group structure <sup>x</sup>  | Mass parent ion | MS <sup>2</sup> spectrum ions (signature ions in bold) | Reference |
|--|-------------------|---|-----------------|--|-----------|
| 2-(benzoyloxy) ethyl                   | -                 |     | 466             | 386  | 33        |
| 3-hydroxy-5-(methylsulfinyl) pentyl    | -                 |     | 482             | 403  |           |
| 4-(β-D-glucopyranosyldisulfanyl) butyl | Diglucothiobeinin |     | 600             | 521  | 33,186    |
| 5-(methylsulfinyl) pentyl              | Glucoalyssin      |     | 450             | 371  |           |
| N-butyl                                | Dihydrogluconapin |    | 374             | 294  |           |
| 4-phenylbutyl                          | Glucoamoracin     |   | 450             | 371  | 28        |
| 7-(methylsulfinyl) heptyl              | Glucoibarin       |   | 494             | 414  |           |
| Ethyl                                  | Glucolepiidin     |  | 346             | 266  |           |



|                              |                         |  |     |   |           |
|------------------------------|-------------------------|--|-----|---|-----------|
| 2-phenylethyl                | Gluconasturtiin         |    | 422 | 343   |           |
| 1-methylethyl                | Glucoputranjivin        |    | 360 | 280   | 28        |
| 4-(methylsulfinyl)-3-butenyl | Glucoraphenin           |    | 434 | 354   |           |
| Dimeric 4-mercaptobutyl      | DMB                     |    | 811 | 731, 569, <b>405</b>                                      | 21,33,186 |
| 4-mercaptobutyl              | Glucosativin            |    | 406 | <b>259</b> , 209, 194, 138<br>97, 96                      |           |
| 4-hydroxy-3-indolymethyl     | 4-Hydroxyglucobrassicin |   | 463 | 383, <b>285</b> , 275, 267,<br><b>259</b> , 240           | 33,178    |
| 4-(methylthio) butyl         | Glucoerucin             |  | 420 | 340, 291, 275, <b>259</b> ,<br>242, 227, 195, 178,<br>163 |           |

|                           |                          |  |     |   |            |
|---------------------------|--------------------------|--|-----|---|------------|
| 4-hydroxybenzyl           | Glucosinalbin            |    | 424 | 344, 291, 275, 261,<br><b>259</b> , 246, 231, 228,<br>182 | 33,178     |
| (R,S)-2-hydroxy-3-butenyl | Progoitrin/epiprogoitrin |    | 388 | 332, 308, 301, 275,<br>259, 210, 195, 136                 |            |
| 3-indolymethyl            | Glucobrassicin           |    | 447 | 275, <b>259</b> , 251, 205                                | 33,178,187 |
| 1-methylpropyl            | Glucocochlearin          |    | 374 | 294   | 85,186     |
| 2-methylbutyl             | Glucojiaputin            |    | 388 | 308   |            |
| 5-(methylsulfonyl) pentyl | Glucoerysienin           |   | 466 | 386   | 28,33      |
| 3-(methylthio) propyl     | Glucoiberverin           |  | 406 | 326, 275, <b>259</b> , 228,<br>145                        | 85,178     |
| 3-butenyl                 | Gluconapin               |  | 372 | 292, 275, <b>259</b> , 227,<br>195, 194, 176              | 28,178     |

|                           |                         |   |     |   |               |
|---------------------------|-------------------------|---|-----|---|---------------|
| Benzyl                    | Glucotrapaeolin         |   | 408 | 328, 275, <b>259</b> , 241,<br>230, 212, 195, 166 |               |
| 1-methoxyindol-3-ylmethyl | Neoglucobrassicin       |   | 477 | 447, <b>466</b> , 284, <b>259</b>                 | 28,178        |
| 2-propenyl                | Sinigrin                |   | 358 | 278, 275, <b>259</b> , 227,<br>195, 180, 162      |               |
| 4-(methylsulfinyl) butyl  | Glucoraphanin           |   | 436 | <b>372</b> , 291, <b>259</b> , 97, 96             | 21,33,178,187 |
| 4-methoxy-3-indolymethyl  | 4-Methoxyglucobrassicin |  | 477 | 291, 275, <b>259</b> , 235,<br>227, 195           | 178, 188      |

<sup>x</sup> = standard GSL molecule according to IUPAC nomenclature

**Table 2:** – List Of Flavonol Compounds Identified In Leaves Of *Eruca* And *Diplotaxis* Species, By LC-MS (Negative Ion Mode).

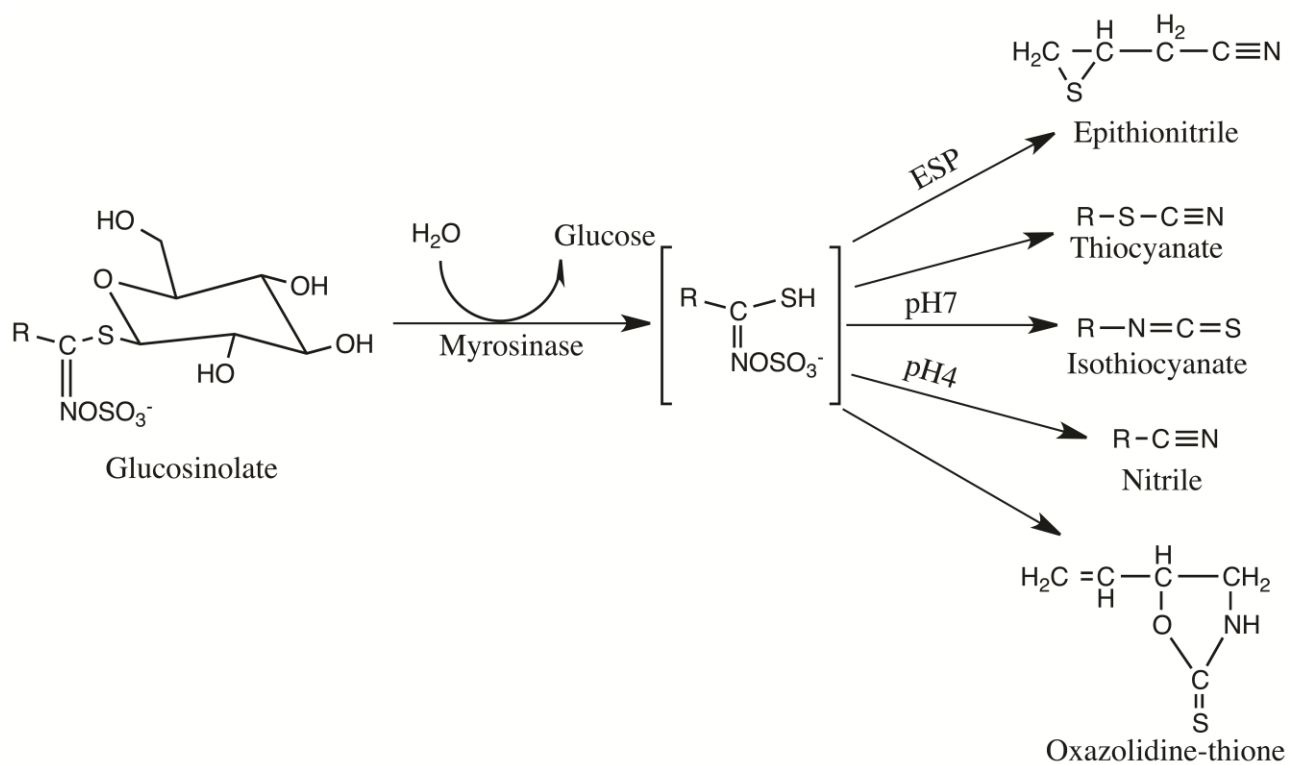
| Flavonol compound <sup>a</sup>          | <i>Eruca</i> <sup>p</sup> | <i>Diplotaxis</i> <sup>p</sup> | Mass parent ion | MS <sup>2</sup> spectrum ions (signature ion in bold) | Reference |
|---|---------------------------|--------------------------------|-----------------|---|-----------|
| I 3,4'-diGlc                            | ✓                         | ✓                              | 639             | <b>477</b>  |           |
| I 3-Glc                                 | ✓                         |                                | 477             | -   |           |
| K 3-(2-Sinp-Glc)-4'-Glc                 | ✓                         |                                | 817             | -   | 18,33     |
| K 3,4'-diGlc                            | ✓                         | ✓                              | 609             | -   |           |
| K 3-Glc                                 | ✓                         |                                | 447             | <b>285</b>  |           |
| Q 3-Glc                                 | ✓                         |                                | 463             | 301   |           |
| K 3-diGlc-7-Glc                         | ✓                         |                                | 771             | 609   |           |
| K 3-Sinp-triGlc-7-Glc                   | ✓                         |                                | 1139            | 977, 771, 609, 429                                    | 33        |
| Q 3,4'-diGlc-3'-(6-Caf-Glc)             |                           | ✓                              | 949             | 787, <b>625</b> , 463, 301                            |           |
| M                                       | ✓                         |                                | 317             | 151   |           |
| Q                                       | ✓                         |                                | 301             | 151   | 189       |
| R                                       | ✓                         |                                | 609             | 300   |           |
| Q 3-(2-Caf-Glc)-3'-(6-Sinp-Glc)-4'-Glc  |                           | ✓                              | 1155            | 993, 831, 787, 669, <b>625</b> , 463, 301             | 18,25     |
| Q 3-(2-Mcaf-Glc)-3'-(6-Sinp-Glc)-4'-Glc |                           | ✓                              | 1185            | 1023, 817, 669, 655                                   |           |
| Q 3-(2-Fer-Glc)-3'-(6-Fer-Glc)-4'-Glc   |                           | ✓                              | 1139            | 977, 639, 463   |           |
| Q 3-(2-Fer-Glc)-3'-(6-Sinp-Glc)-4'-Glc  |                           | ✓                              | 1169            | 1007, 831, 669, 639, 463, 301                         | 18,25,33  |

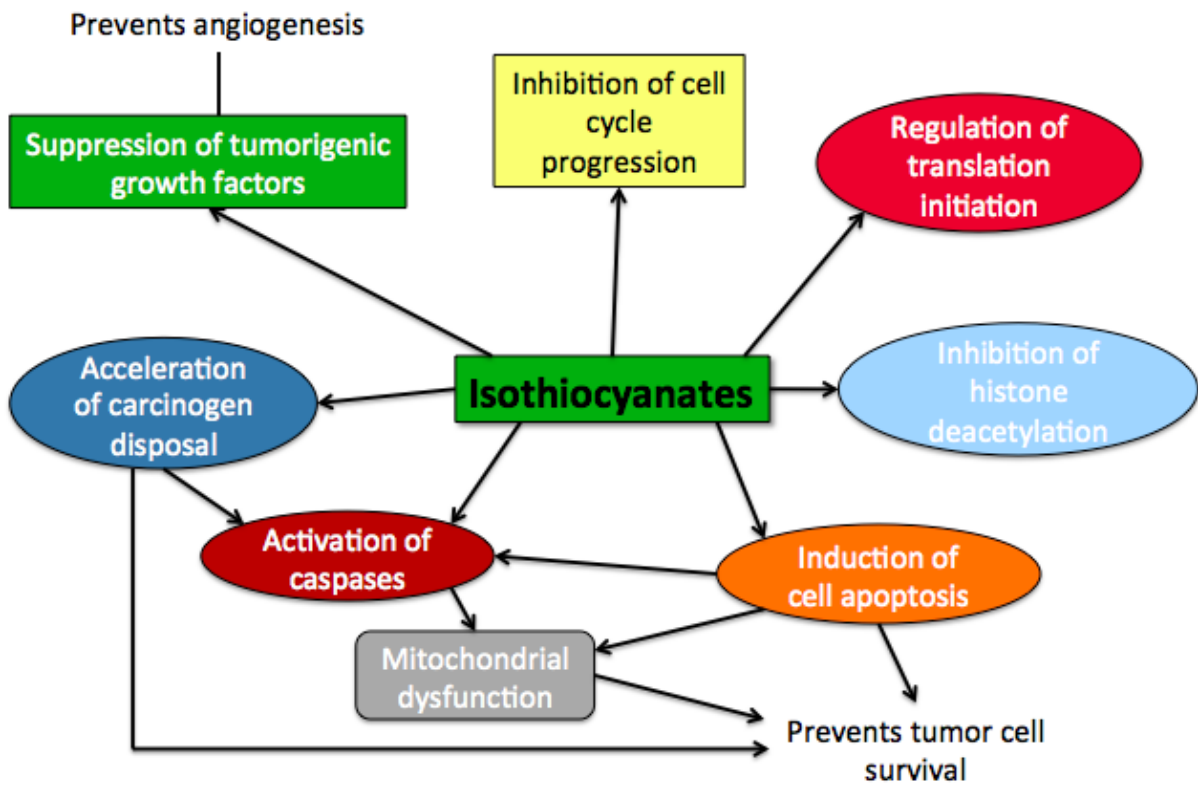
|   |   |      |                          |          |
|---|---|------|--------------------------|----------|
| Q 3-(2-Sinp-Glc)-3'-(6-Sinp-Glc)-4'-Glc | ✓ | 1199 | 1037, 831, 669, 463, 301 |          |
| Q 3,3',4-triGlc                         | ✓ | 787  | 625, 463, 301            |          |
| Q 3,4'-diGlc-3'-(6-Fer-Glc)             | ✓ | 963  | 801, 639, 463, 301       | 18,25,33 |
| Q 3,4'-diGlc-3'-(6-Mcaf-Glc)            | ✓ | 979  | 817, 655, 463, 301       |          |
| Q 3,4'-diGlc-3'-(6- <i>p</i> .Coum-Glc) | ✓ | 933  | 771, 609, 463, 301       |          |
| Q 3,4'-diGlc-3'-(6-Sinap-Glc)           | ✓ | 993  | 831, 669, 463, 301       |          |

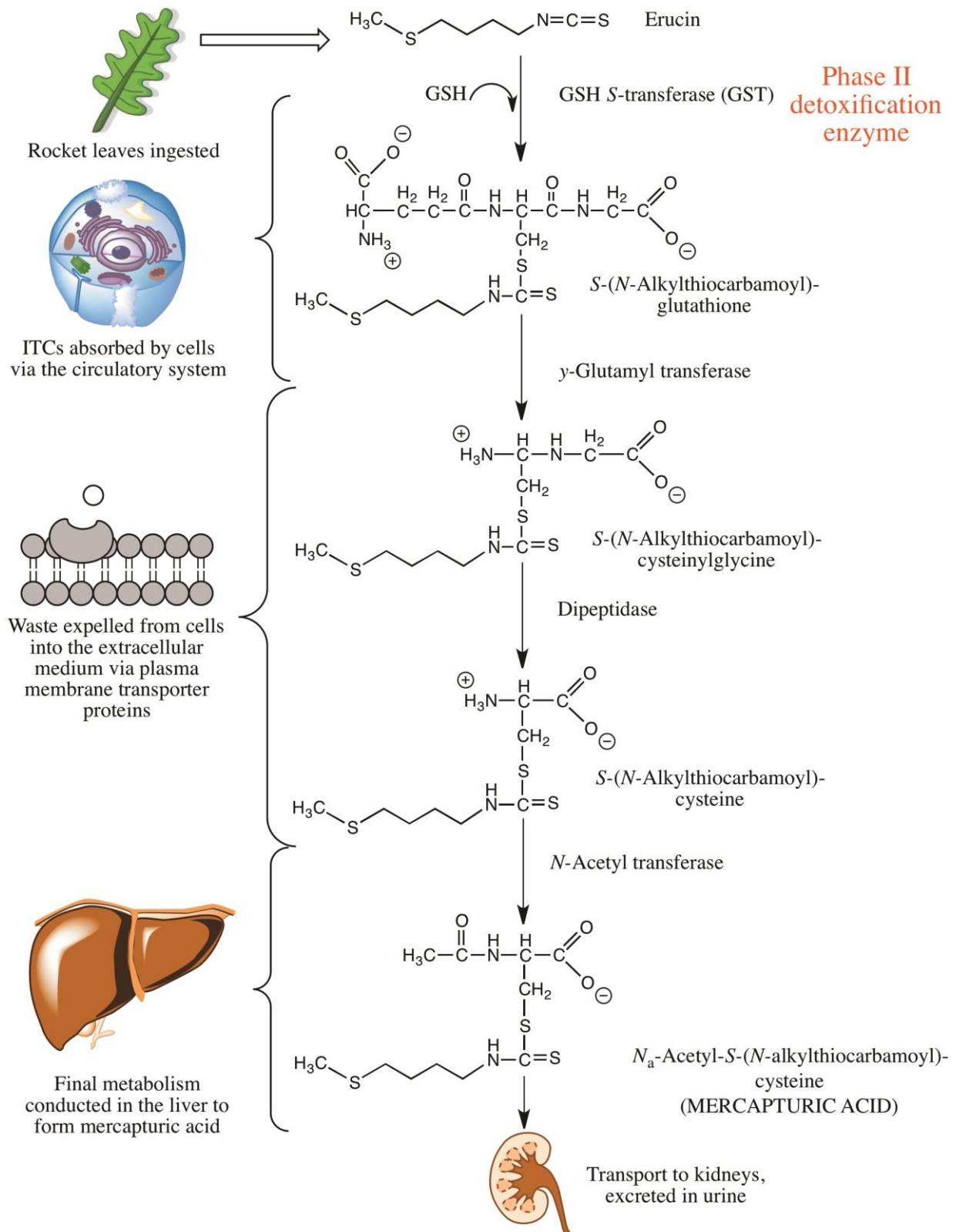
<sup>a</sup> = Abbreviations: Caf, caffeyol; Mcaf, methoxycaffeyol; *p*.Coum, *p*-coumaroyl; Fer, feruloyl; Sinp, sinapoyl; Glc, glucoside;

Q, quercetin; K, kaempferol; I, isorhamnetin; M, myricetin; R, rutin

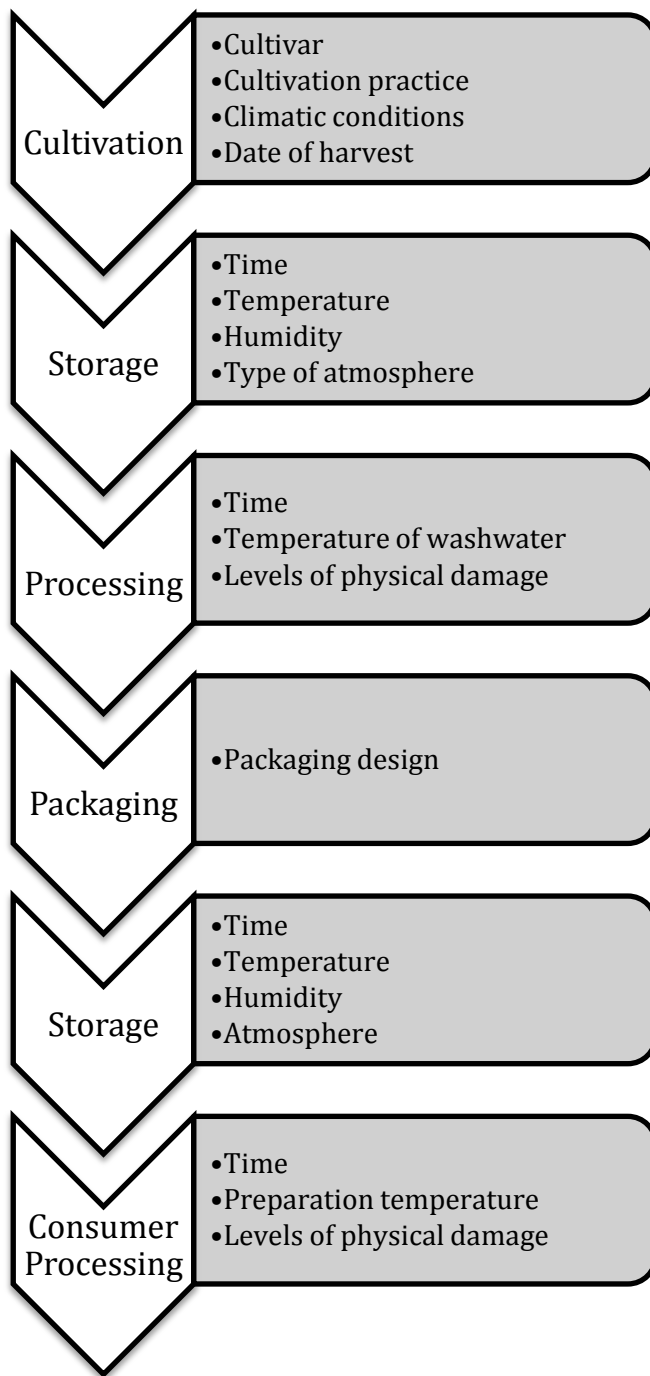
<sup>p</sup> = ✓ compound positively identified in species











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